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基础研究

碱性成纤维生长因子单克隆抗体联合伊立替康抑制小细胞肺癌H223细胞的增殖

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摘要:目的 探讨碱性成纤维生长因子(bFGF)单克隆抗体与伊立替康联合应用体外对抑制小细胞肺癌细胞株增殖和凋亡的作用及可能机制。**方法** CCK8检测bFGF单克隆抗体联合伊立替康对小细胞肺癌株H223增殖的抑制作用。AnneginV-FITC/PI染色流式细胞仪检测新型bFGF单克隆抗体联合伊立替康对小细胞肺癌株H223凋亡的影响。Western blotting分析bFGF-mAb联合CPT-11对AKT和ERK1/2磷酸化水平的影响。**结果** bFGF单克隆抗体、伊立替康剂量依赖性抑制小细胞肺癌株H223的增殖,bFGF单克隆抗体组、伊立替康组及bFGF单克隆抗体联合伊立替康组对小细胞肺癌株H223的增殖抑制率分别为18.73%、21.96%、54.30%,联合用药组抑制率明显高于单药组($P<0.05$)。bFGF单克隆抗体、伊立替康剂量依赖性诱导小细胞肺癌株H223的凋亡,bFGF单克隆抗体组、伊立替康组及bFGF单克隆抗体联合伊立替康组诱导小细胞肺癌株H223的早期凋亡率分别为2.7%、4.3%、6.5%,联合用药组凋亡率明显高于单药组($P<0.05$)。bFGF单克隆抗体组、CPT-11组及bFGF单克隆抗体联合CPT-11组可明显抑制p-AKT蛋白和p-ERK1/2蛋白的水平,差异有显著性,而对AKT、ERK1/2蛋白则影响不大。bFGF抗体联合CPT-11一方面通过抑制p-AKT蛋白和p-ERK1/2蛋白的水平来抑制肿瘤细胞的增殖和转移。**结论** bFGF单克隆抗体联合伊立替康对小细胞肺癌株H223具有协同抑制作用,其机制与抑制细胞增殖和促进细胞凋亡有关。信号通路分析结果表明bFGF单克隆抗体联合伊立替康能有效阻断与bFGF相关的MAPK/ERK和PI3K/AKT信号通路。

关键词:碱性成纤维生长因子;bfgf单克隆抗体;伊立替康;小细胞肺癌;增殖;凋亡;MAPK/ERK1/2;PI3K/AKT

Effect of basic fibroblast growth factor antibody combined with irinotecan on proliferation and apoptosis of small cell lung cancer H223 cells *in vitro*

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Abstract: Objective To study the synergistic inhibitory effects of basic fibroblast growth factor (bFGF) monoclonal antibody (bFGF mAb) and irinotecan on the proliferation of small cell lung cancer H223 cells. **Methods** CCK-8 assay and flow cytometry were used to assess the effects of bFGF mAb combined with irinotecan on the proliferation and apoptosis of H223 cells, respectively. Western blotting was performed to analyze the effect of bFGF-mAb combined with irinotecan on AKT and ERK1/2 phosphorylation in the cells. **Results** Both bFGF mAb and irinotecan alone inhibited H223 cell proliferation in a dose-dependent manner ($P<0.05$). The inhibitory rate was significantly higher in H223 cells treated with bFGF mAb + irinotecan (54.30%) than in cell treated with bFGF mAb (18.73%) or irinotecan (21.96%) alone ($P<0.05$). Both bFGF mAb and irinotecan induced H223 cell apoptosis in a dose-dependent manner ($P<0.05$), and the combined treatment resulted in a significantly higher early apoptosis rates (6.5%) than treatment with bFGF mAb (2.7%) or irinotecan (4.3%) alone ($P<0.05$). bFGF mAb and irinotecan, either alone or in combination, significantly inhibited the levels of p-AKT protein and p-ERK1/2 protein without obviously affecting AKT and ERK1/2 protein levels. **Conclusion** bFGF mAb and irinotecan produce synergistic inhibitory effects on small cell lung cancer H223 cells by suppressing proliferation and promoting apoptosis of the cells, and can effectively block the MAPK/ERK and PI3K/AKT signaling pathways associated with bFGF.

Keywords: basic fibroblast growth factor; monoclonal antibody; irinotecan; small cell lung cancer; proliferation; apoptosis; MAPK/ERK1/2; PI3K/AKT

小细胞肺癌是一种极具侵袭性的恶性肿瘤,常预后不良[1],传统的手术及放化疗在患者的治疗上一直无突破。最近几年生物靶向药物的出现在非小细胞肺癌的治疗中已经有了质的的飞跃,而在小细胞肺癌中仍然停滞不前。纵观肿瘤的复发转移与血管生长密切相关,从而使得抗血管生成的生物靶向药物与化疗药物的联合应用奠定了理论基础,相关药物的研究更是突飞猛进[2-3]。

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碱性成纤维细胞生长因子(bFGF)是诱导肿瘤细胞增殖的生长因子,在多种恶性肿瘤中阳性表达,有抗肿瘤血管生成的作用。bFGF单克隆抗体通过与fgfr的结合而达到显著抑制肿瘤的生长及转移,bFGF在非小细胞肺癌亦有研究[4],但在小细胞肺癌中研究较少。而且bFGF是多种肿瘤的耐药因素,主要机制是抑制化疗药物诱导的细胞凋亡使多数化疗药耐药[5-6]。伊立替康是拓扑异构酶I的抑制剂,在日本的临床研究中IP(伊立替康+顺铂)疗效优于EP(依托泊苷+顺铂)[7],因此本研究选用本实验室纯化的新型bFGF单克隆抗体联合伊立替康应用于小细胞肺癌细胞的治疗,理论上有协同作用。在国内外还未见报道。通过新型bFGF单克隆抗体抑制肿瘤血管看能否抑制小细胞肺癌的生长,缓解化疗药物的耐药性,探索两者在体内的协同抑及可能机制,为bFGF单克隆抗体靶向治疗小细胞肺癌提供实验依据。

1 材料和方法

1.1 细胞株

H223小细胞肺癌珠来自暨南大学抗体工程研究中心冻存,本实验用于治疗的bFGF单克隆抗体来自中山康方生物有限公司。

1.2 主要试剂及仪器

青霉素和链霉素双抗(Invitrogen);10%胎牛血清(S/N:16000-044;Gibco);96孔板中(Corning, USA);DMEM培养基和胎牛血清FBS购自Gibco公司;Akt、ERK1/2、t-/p- Akt、t-/p- ERK1/抗体(Cell Signaling Technology);Super-GL ECL超敏发光液(诺伦生物);裂解液(诺伦生物);PVDF Transfer Membrane (Millipore);转膜滤纸(bio-rad)KODAK X-Omat BT Film(Kodak);X光片显影液;X光片定影液(Kodak);HRP-cnjugated goat anti- mouse IgG(抗体使用浓度1:1000);HRP-cnjugated goat anti- rabbit IgG(抗体使用浓度1:1000);GADPH(Sigma,抗体使用浓度1:5000);CCK-8试剂盒(碧云天)。伊立替康注射液(恒瑞药业)。酶标仪(Multiskan)。PowerPac™HC电泳仪(bio-rad);VE-180垂直电泳槽(天能);Semi-Dry Transfer Cell (bio-rad);DY-B1脱色摇床(沪西仪器);Olympus-80 ℃超低温冰箱;低温超速离心机和低速离心机(Hitachi);紫外分光光度计和凝胶成像系统(Lumat LB 9507);荧光倒置相差显微镜(Olyplus);CO_2培养箱(Heraeus);奥德赛条带扫描仪(S/N ODY-2792 model;9120);超净台(苏州净化设备)。

1.3 小细胞肺癌细胞增殖抑制实验

取处于对数生长期的小细胞肺癌细胞,调整细胞密度至4×10^4/mL,每孔100 μL接种到96孔细胞培养板中,37 ℃培养24 h后分组加药:(1)bFGF单克隆抗体组:终质量浓度为50、100和20 μg/mL;(2)伊立替康组:将伊立替康溶于NS中,终质量浓度为5、10、20 μg/mL;(3)bFGF单克隆抗体联合伊立替康组:选用质量浓度为100 μg/mL的bFGF单克隆抗体分别与5、10、20 μg/mL的伊立替康联合作用,每孔伊立替康终质量浓度与伊立替康组终质量浓度相同;(4)空白对照组:仅加入100 μL培养液。培养72 h,加入10 μL/孔的CCK-8试剂,继续培养1.5 h,于酶标仪读取吸光度A_{450}。每浓度设3个复孔,实验重复3次。IR=(1-药物组A)/(空白对照组A)×100%。

1.4 小细胞肺癌细胞凋亡抑制实验

H223细胞被覆盖在6孔细胞培养板中,培养24 h后分组加药,方法见1.3,一起孵化96 h。细胞凋亡的片段用FITC标记的膜联蛋白V和碘化丙啶 propodium(PI)标记,在室温下孵化15 min后样本用流式细胞仪检测。

1.5 Western blotting分析bFGF-mAb联合CPT-11对AKT和ERK1/2磷酸化水平的影响

H223细胞被覆盖在6孔细胞培养板中,培养24 h后,待细胞贴壁后,移液器吸掉完培,加入2 mL的DMEM基培,继续培养12 h后,分组加药,方法见1.3,一起孵化1 h。每个组细胞样本加入300 μL细胞裂解液,用枪混匀使其完全裂解,将裂解物移至新的离心管中。直接取3 μL样本加入10 μL 2×SDS-PAGE loading buffer,混匀,100 ℃加热处理5 min,冰上冷却,12 000 *g*离心5 min去除不溶性沉淀。样品使用10% SDS-PAGE分离,每孔最大上样量为20 μL。电泳结束后,将PVDF膜在甲醇中浸泡1 min,再使用Transfer Buffer浸泡凝胶、滤纸和在甲醇中浸泡过的PVDF膜一起4 ℃放置10 min,然后制备转移三明治。本实验使用Semi-Dry Cell进行半干电泳转移,转移条件为30 mA 60 min。使用Blocking Buffer封闭转印膜4 ℃封闭过夜,第2天用1×TBST洗涤3次, 15 min/次。加入稀释好的一抗,37 ℃温育2 h。用1×TBST洗涤4次, 10 min/次。加入稀释好的二抗,37 ℃温育2 h。用1×TBST洗涤4次,10 min/次。用Super-GL ECL超敏发光液进行化学发光检测,并对X光片曝光。经显影定影处理后,晾干的胶片最后用凝胶成像分析系统拍照,本实验采用的是Gel-Pro Analyzer软件来分析处理。

1.6 统计学处理

计量资料采用均数±标准差表示,运用SPSS 18.0软件 对相关数据进行统计学处理,两组间计量资料比较采用t检验,率的比较采用χ检验,以P<0.05表示差异有统计学意义。

2 结果

2.1 bFGF单克隆抗体单用及联合CPT-11抑制小细胞肺癌细胞的增殖

浓度为5、10、20 μg/mL的伊立替康与100 μg/mL的bFGF单克隆抗体联合作用于小细胞肺癌细胞,其抑

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制作用明显强于两者单独作用(P<0.05,图1)。

2.2 bFGF单克隆抗体单用及联合CPT-11诱导小细胞肺癌细胞的凋亡

浓度为5、10、20 μg/mL的伊立替康与100 μg/mL的bFGF单克隆抗体联合作用于小细胞肺癌细胞,其诱导作用明显强于两者单独作用(P<0.05,图2)。

2.3 Western blotting检测bFGF抗体组,CPT-11组及bFGF抗体联合CPT-11组对H223中p-AKT蛋白和p-ERK1/2蛋白的影响

bFGF单克隆抗体组、CPT-11组及bFGF单克隆抗体联合CPT-11组可明显抑制p-AKT蛋白和p-ERK1/2蛋白的水平,差异有显著性(P<0.05),而对AKT、ERK1/2蛋白则影响不大(P>0.05,图3)。

3 讨论

恶性肿瘤的复发转移同血管的新生密不可分,而肿

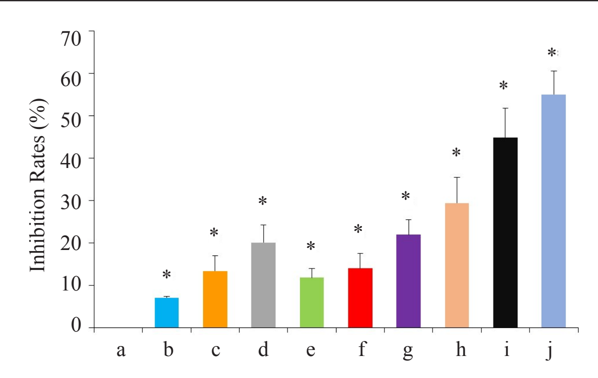
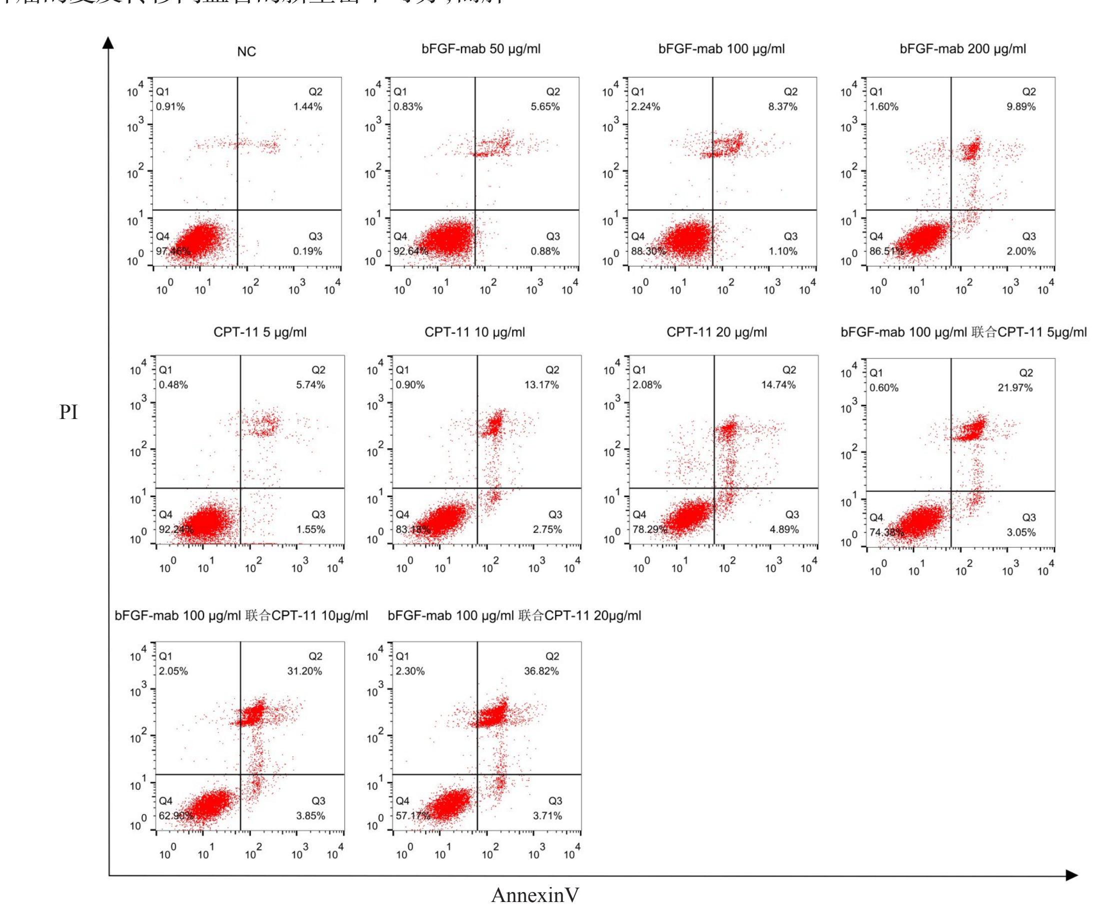


图1 bFGF单克隆抗体单用及联合CPT-11对小细胞肺癌细胞增殖的影响

Fig.1 Effects of bFGF mAb and CPT-11, alone or in combination, on the proliferation of small cell lung cancer cells. *P<0.05 *vs* NS. a: NS; b: bFGF-mab 50 μg/mL; c: bFGF-mab 100 μg/mL; d: bFGF-mab 200 μg/mL; e: CPT-11 5 μg/mL; f: CPT-11 10 μg/mL; g: CPT-11 20 μg/mL; h: bFGF-mab 100 μg/mL+CPT-11 5 μg/mL; i: bFGF-mab 100 μg/mL+CPT-11 10 μg/mL; j: bFGF-mab 100 μg/mL+CPT-11 20 μg/mL.



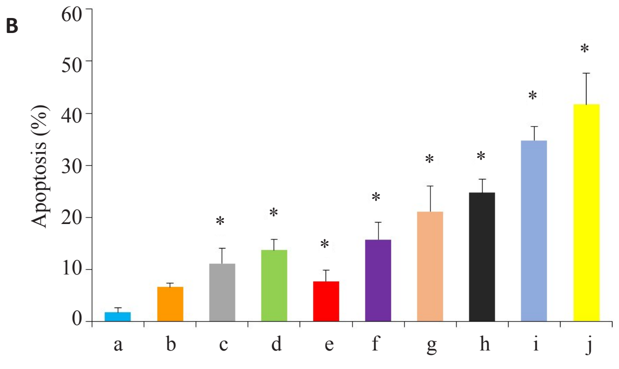


图2 bFGF单克隆抗体单用及联合CPT-11对小细胞肺癌细胞凋亡的影响

Fig.2 Effect of bFGF mAb and CPT-11, alone or in combination, on apoptosis rate of H223 cells detected by FACS method. ***A***: Flow cytometry; ***B***: Quantitative analysis of the results. *P<0.05 *vs* NS. a: NS; b: bFGF-mab 50 μg/mL; c: bFGF-mab 100 μg/mL; d: bFGF-mab 200 μg/mL; e: CPT-11 5 μg/mL; f: CPT-11 10 μg/mL; g: CPT-11 20 μg/mL; h: bFGF-mab 100 μg/mL+CPT-11 5 μg/mL; i: bFGF-mab 100 μg/mL+CPT-11 10 μg/mL; j: bFGF-mab 100 μg/mL+CPT-11 20 μg/mL.

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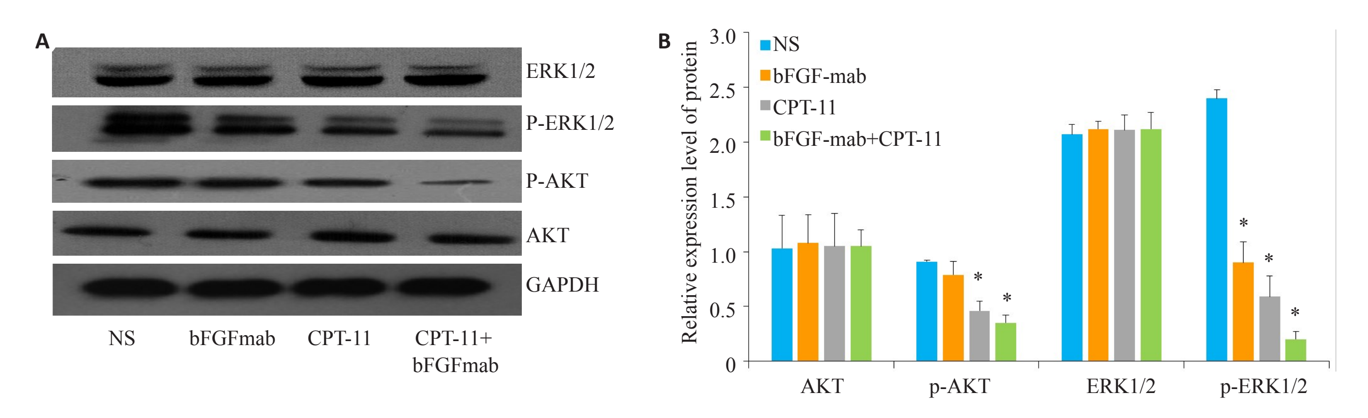


图3 Western blotting检测bFGF抗体组,CPT-11组及bFGF 抗体联合CPT-11组对H223中p-AKT 蛋白和p-ERK1/2蛋白的影响

Fig.3 Western blotting for detecting the effect of bFGF mAb and CPT-11, alone or in combination, on p-AKT protein and p-ERK1/2 protein in H223. *A*: Western blotting; *B*: Quantitative analysis of the results. **P*<0.05 *vs* NS.

瘤的血管的形成主要受血管内皮生长因子(VEGF)和bFGF的调控[8],bFGF本身是一种多肽细胞生长因子,不但影响细胞的分裂增殖,而且在伤口的修复及组织的再生上发挥作用。小细胞肺癌是一种侵袭性极高的恶性肿瘤,易远处转移且预后不良[9-10]。既往研究表明[11],血清中的bFGF高表达常与小细胞肺癌患者的不良预后相关,可能通过bFGF上调抗凋亡蛋白的表达诱导细胞自我保护从而促进部分肿瘤耐药。FGFR2能通过直接与bFGF结合减少恶性细胞转移。近来有研究提示FGFR2蛋白过表达在未分化的恶性肿瘤中与分化好的恶性肿瘤有明显差异[12]。而不同恶性肿瘤的FGFR2蛋白过表达存在明显差异,且与生存率差显著相关,常提示预后不良[13-19]。作为一种细胞因子一方面通过刺激VEGF的表达影响肿瘤血管内皮细胞的增殖,另一方面通过自分泌及旁分泌直接促进肿瘤细胞增殖,进而导致新生血管形成。BFGF单克隆抗体通过与受体结合从而阻止细胞增殖抑制血管生成[20]。体内外试验证实[21-22] BFGF能促进多种因子分泌,有报道[23]BFGF/FGFR可介导恶性肿瘤的自我保护机制从而促进肿瘤生长。亦有试验报道[24-26]FGF/FGFR信号通路介导了肺癌的体内外EGFR治疗的抵抗。而治疗性的bFGF单克隆抗体与特定的bFGF受体或fgfr异构体有高度的特异性,通过依赖的抗体或补体抗肿瘤细胞毒作用增强机体的免疫作用从而达到自身的抗肿瘤活性[11]。本实验的CKK8法结果提示BFGF单克隆抗体抑制小细胞肺癌细胞的增殖,200 μg/mL的BFGF单克隆抗体抑制率为18.73%,而100 μg/mL bFGF联合伊立替康20 μg/mL组抑制率高达54.30%。既往研究显示BFGF能使较多肿瘤产生对化疗药物的抵抗,主要原因是抑制化疗药物诱导的细胞凋亡[27],伊立替康是拓扑异构酶Ⅰ的抑制剂,因抗肿瘤活性强,其活性代谢产物7-乙基-10羟基喜树碱(SN-38)在体内与拓扑异构酶Ⅰ(TopoⅠ)结合,阻止DNA链重新组装,促使DNA双链断裂,从而干扰和抑制DNA的复制以抑制细胞的有丝分裂。CPT-11在SCLC的治疗中效果显著,并且与VP-16、DDP没有交叉耐药,本研究中的AnneginV-FITC/PI法检测细胞凋亡结果提示单克隆抗体200 μg/mL的凋亡率是2.7%,而单克隆抗体100 μg/mL联合伊立替康20 μg/mL组凋亡率高达6.5%,进一步证明在单克隆抗体BFGF联合伊立替康组在抑制肿瘤方面的协同作用。既往研究[28]显示bFGF等细胞因子可能通过激活AKt/PKB通路阻断生长信号途径从而抑制细胞增殖,而CPT-11亦可抑制AKt/PKB通路,因此两者的协同作用可能与抑制AKt/PKB通路激活有关。本实验结果提示bFGF单克隆抗体联合伊立替康通过抑制p-AKT蛋白、p-ERK1/2蛋白的水平磷酸化来抑制肿瘤细胞的增殖和转移。bFGF抗体联合伊立替康可能通过Ras-Raf-MAP激酶激活Ras启动,产生导致的多步磷酸化级联激活MAPKs,ERK1和ERK2而达到抗肿瘤的目的[29-30],同时通过抑制另一个重要目标FGFR信号是磷脂酰肌醇3-激酶(P13K)和下游蛋白丝氨酸/苏氨酸激酶AKT的表达来达到抗肿瘤目的。后一种蛋白激酶转导分子信号触发细胞生长的关键步骤。小细胞肺癌占肺癌发病率的15%~25%,肿瘤倍增时间短,侵袭能力强,绝大多数患者诊断时已存在血行和淋巴结转移[31],预后差,传统治疗模式存在的耐药及毒性严重的问题迫切需要新的治疗方法,本试验的结果提示BFGF单克隆抗体联合CPT-11在抑制小细胞肺癌中的协同作用为小细胞肺癌的治疗提供了新的思路,有关两者协同抑制肿瘤细胞增殖及血管新生的细胞因子表达及具体机制还需进一步研究探讨。

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